1979

Bacteriophage Populations in Wastewater Effluent

Guy William Lawrence
Eastern Illinois University

PAPER CERTIFICATE #2

TO: Graduate Degree Candidates who have written formal theses.

SUBJECT: Permission to reproduce theses.

The University Library is receiving a number of requests from other institutions asking permission to reproduce dissertations for inclusion in their library holdings. Although no copyright laws are involved, we feel that professional courtesy demands that permission be obtained from the author before we allow theses to be copied.

Please sign one of the following statements:

Booth Library of Eastern Illinois University has my permission to lend my thesis to a reputable college or university for the purpose of copying it for inclusion in that institution's library or research holdings.

27 April, 1979
Date

I respectfully request Booth Library of Eastern Illinois University not allow my thesis be reproduced because _____

_____

_____

_____ _____
Date Author

pdm

BACTERIOPHAGE POPULATIONS IN WASTEWATER EFFLUENT

(TITLE)

BY

GUY WILLIAM LAWRENCE

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1979
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

27 Apr, 1979
DATE

April 27, 1979
DATE

ACKNOWLEDGEMENTS

The author wishes to express his most sincere gratitude to Dr. William A. Weiler for his interest, ideas, encouragement and unerring guidance in the writing of this thesis and in the author's effort to acquire the Master of Science degree.

Special thanks are extended to the author's graduate committee, Dr. John M. Speer and Dr. U. Douglas Zimmerman, for their constructive criticism in the preparation of this paper and help throughout his course of study at Eastern Illinois University.

Further recognition goes to Mr. L. E. Crofutt and Dr. Kandy Baumgardner for providing the necessary bacterial cultures and for their help in the literature search.

Finally, to his parents, sincerest thanks are extended, for without their moral and financial support, all of this would not be possible.

TABLE OF CONTENTS

LIST OF TABLES

ABSTRACT

Lawrence, Guy William. M.S., Eastern Illinois University, May 1979. Bacteriophage Populations in Wastewater Effluent. Major Professor: Dr. William A. Weiler.

Effluent samples were taken at various stages of sewage treatment at a local wastewater treatment plant. Primary, secondary, tertiary and post-chlorinated effluents were sampled. The agar overlay method was used to determine the numbers of plaque-forming bacteriophage particles on thirty-six species of enteric bacteria from the Eastern Illinois University stock culture collection. Twenty-three pure culture phage isolates were prepared from selected plaques and their host range specificity was determined. For every host organism tested, a fifty percent or greater reduction of bacteriophage was evident through the sewage treatment process. Results obtained from investigation of host range specificity indicate that potential exists for phage-mediated transduction to occur in a natural environment.

INTRODUCTION

Researchers have generally recognized the presence of bacteriophages in sewage wastewater. Particularly well-known are those viruses which attack members of the bacterial family Enterobacteriaceae. Since the discovery of bacterial viruses in 1915 by Twort and subsequent observations in 1917 by d'Herelle, the bactericidal properties of bacteriophages have been well documented. Some bacterial viruses are known to act as carriers of bacterial genes from one cell to another in the process called transduction. Transduction is known to occur in very few groups of bacteria. It was first reported in 1952 by Zinder and Lederberg, who used a strain of *Salmonella typhimurium*. Since this discovery, the occurrence of phage-mediated transduction has been reported in strains of *Shigella*, *Pseudomonas*, *Staphylococcus*, *Proteus*, *Bacillus subtilis* and *Escherichia coli*. Most such observations have been made with pure cultures *in vitro;* little research has been conducted on mixed cultures in natural environments.

The initial purpose of the research reported here was to enumerate the enteric bacteriophages present at various stages in the sewage treatment process at a local wastewater treatment plant. Subsequent emphasis was placed on the potential of such populations to facilitate transduction among the mixed enteric bacteria present in sewage effluent.

There were three questions in mind at the outset of experimentation: (1) What are the effects of sewage treatment, particularly chlorination,

on the enteric bacteriophage populations present in a wastewater treatment plant? (2) Do the bacteriophage populations present in sewage effluent exhibit limited or broad host ranges? (3) Is transduction possible in wastewater?

The reasoning behind the experimentation was relatively straightforward. Transduction is possible (1) if there are sufficiently large numbers of enteric bacteriophages present in sewage, and (2) if the enteric bacteriophages present exhibit a sufficiently broad host range.

HISTORICAL

Bacteriophage Ecology

There appears to be little research in the literature concerning the ecological relationship between bacteria and their viruses in natural environments. Although any given bacteriophage may be highly host specific, it is probable that every known type of bacterium serves as host to one or more phages. Some of the common sources of bacterial viruses are soil, sewage, feces and any material in which large numbers of bacteria are present.

In describing the action of phages on bacteria, several terms are used (Jacob *et al.*, 1953, as reported by Anderson, 1957). Lysogeny is the ability of a bacterial strain to produce phage and to transmit this property to its progeny. When the bacterial cell divides, each daughter cell will carry the latent phage infection (Lisbonne and Carrere, 1922, as reported by Hewitt, 1953; d'Herelle, 1926; Burnet and Lush, 1936). Since lysogeny is inherited, it has the properties of a genetic character. In addition, lysogenic bacteria are resistant to the phages they carry. These lysogenic bacteria are the probable reservoir of bacteriophages in nature and may account for the presence of high numbers of phage in soil and sewage.

Lysogenization simply refers to the production of the lysogenic state. Bacteriophages which are capable of attaining this relationship with specific bacterial hosts are known as temperate phages. They do

possess the ability to lyse other strains of bacteria usually referred to as indicator strains. A prophage is the latent form of a bacteriophage while it is being carried by a lysogenic bacterium. A virulent phage is one which does not possess the ability to form a lysogenic complex with a host cell and can undergo only lytic reproduction.

A bacteriophage may influence a population of bacteria in several ways (Anderson, 1957): (1) A virulent phage may eliminate sensitive cells, thereby causing replacement of a population with phage-resistant mutants. (2) A temperate phage may destroy a fraction of a bacterial population and lysogenize the remainder. (3) Virulent and temperate phages are both capable of transduction of genetic material with or without lysogenization of the initial host (Anderson, 1957).

Many problems arise in attempting to study bacteriophage ecology. The greater the size of the ecosystem, the greater the number of heterogeneous bacteria and bacteriophages which must be considered. It appears much more feasible to study this type of microbial ecology on a small scale basis. As a result, pure culture studies abound in the literature.

The Bacteriophage Growth Cycle

A typical bacteriophage particle consists of a head and a tail. The head represents an outer protein shell that contains a mass of nucleic acid, usually deoxyribonucleic acid (DNA), but in some isometric phages ribonucleic acid (RNA) may be present (Lindberg, 1973). The protein coat surrounding the head of the virus serves to protect the enclosed nucleic acid. Many bacteriophages examined resemble tadpoles with long tails and a head which may be spherical, cylindrical or polyhedral. The phage tail varies tremendously in complexity from one phage to another. Some phages were originally thought to be tailless spheres but were

later found to be polyhedral shapes with a rudimentary tail (Williams and Fraser, 1953). The shapes and relative proportions of the head and tail seem to vary within wide limits among different phages. In numerous instances, the tail proves to be a specialized organelle for attachment to bacterial cells; it is very probable that all phages possess this structure (Anderson, 1953). In the most extensively studied bacteriophages, the coliphages, the tail is known to consist of at least three parts: a hollow core, a contractile sheath and a hexagonal-shaped terminal baseplate which may have prongs, tail fibers or both attached to it (Jawetz *et al.*, 1974).

The reproductive cycle of a bacteriophage is generally divided into four arbitrary stages (1) adsorption of the phage particle to a susceptible bacterial cell; (2) penetration of the bacterial cell wall by the phage; (3) the intracellular replication of the virus; and (4) the lysis of the host cell and release of phage progeny.

The growth cycle of a typical bacteriophage begins with its adsorption to a bacterial cell. The rate of attachment depends not only on the concentration of phage and bacteria but also on the ionic environment, temperature, pH and organic cofactors (Adams, 1959; Stent, 1963). If adsorption does not take place, the infectious process cannot continue and the host bacterial cell will continue to multiply as if there were no phage present. The adsorption process is very specific. Different bacterial strains are highly specific with regard to the phages which they will adsorb.

Penetration is the second step in the infectious process and little appears to be known about it. Hershey and Chase (1952) provided convincing evidence of penetration using T2 coliphage labeled with radioactive phosphorous and sulfur. Since phage progeny are formed intracellularly

and released on lysis of the host bacterial cell, it is assumed that the tail of the bacterial virus enzymatically pierces the cell wall of the host. A clue as to how penetration occurs involved the discovery that the tails of the T-even phages contain a lysozyme-like enzyme which acts on the lipopolysaccharide layer of the bacterial cell wall (Barrington and Kozloff, 1954). The protein tail of the phage is hollow and serves as an exit for the DNA from the bacteriophage head to the cytoplasm of the bacterial cell. The nucleic acid at this stage is often referred to as vegetative phage in order to distinguish it from mature, infective phage particles (Doermann, 1953).

Following penetration and injection of viral nucleic acid, new vegetative phages are produced. Synthesis of phage protein and phage DNA commence. Protein subunits of the phage head and tail self-assemble to form mature progeny virions (infectious phage particles). Phage maturation involves combination of phage nucleic acid with the protein coat (Jawetz *et al.*, 1974).

Following intracellular replication of the virus, the host cell will lyse due to weakening of the cell wall by phage lysozyme, liberating up to several hundred infectious phage particles. Lysis is not dependent on the accumulation of mature phage particles because lysis will occur even when phage multiplication is interrupted by chemicals such as cyanide (Cohen, 1949) or proflavine (Foster, 1948). The only method of temporarily interrupting the lytic process is chilling, which slows down all enzymatic reactions (Adams, 1959).

The period of time from attachment of a bacteriophage to a host cell until the release of the progeny is termed the latent period. The number of phage actually released per cell at the end of the latent period is termed the burst size. Many temperate phages are capable of

being reduced to prophage as an alternative to producing lysis of the host cell. The bacterial cell in this case is termed lysogenic and, after an indeterminate number of cell divisions, one of its daughter cells may lyse and liberate infective phage particles. Occasionally, a lysogenic bacterium may lose its prophage and remain viable as an uninfected cell. The cause of this loss is unknown (Jawetz *et al.*, 1974).

Since the scope of this study was to examine the host range specificity of bacteriophages in a natural environment, closer attention must be given to the adsorption process since it is the stage during which host specificity is probably determined. D'Herelle (1926) showed that bacteria which are not able to adsorb a given phage cannot serve as hosts for its multiplication. He observed that some bacteriophages were highly specific attacking only certain strains in a single species of bacterium, while other phages possessed the ability to attack bacteria in different genera. Lazarus and Gunnison (1947) observed a *Pasteurella pestis* (*Yersinia pestis*) phage which would also lyse some *Salmonella* species and some *Shigella* species. Phage strains capable of attacking only a single bacterial species were termed "monovalent" and those which could attack two or more bacterial species were called "polyvalent" (Kalmanson and Bronfenbrenner, 1942). Since infection by phage tends to be highly host-specific and to depend on the structure of the bacterial surface, random collision between phage and bacterium must be followed by a precise attachment of the phage tail to the cell wall of a susceptible bacterium. Purified cell walls of sensitive bacteria are found to adsorb phage as actively as living bacteria. Protoplasts, which lack cell walls, do not adsorb phage. Yet, protoplasts may support phage growth if infection occurred before removal

of the bacterial cell wall (Weibull, 1953; Zinder and Arndt, 1956). Therefore, the bacterial cell wall must contain phage-specific receptor sites that are able to undergo a spontaneous and irreversible chemical reaction with the attachment organelles of the virus particle. The bacterial surface may be thought of as a mosaic of various phage receptor sites, each receptor being capable of forming a highly specific bond with the phage type to which it is receptive.

Nearly every exposed structure on the surface of a bacterial cell or extending from it can act as (or include) phage receptor sites (Lindberg, 1973). This includes the outer membrane of Gram-negative bacteria, the cell wall of Gram-positive bacteria, the capsular layer of Gram-negative bacteria and, if present, the flagella and pili (Lindberg, 1973). The phage receptor may be a capsular polysaccharide or it may be located on or in the rigid cell wall (Salton, 1960). The only extensive studies on the chemical nature of bacteriophage receptors appear to be those of Weidel and his co-workers who showed that aldoheptoses and amino sugars are common to the cell wall receptors of *E. coli B* (Weidel, *et al.*, 1954; Weidel, 1955, as reported by Salton, 1960).

Phages provided with different organelles for adsorption differ in their mode of attachment to the bacterial cell. At least three methods of attachment can be recognized (Lindberg, 1973): (1) Large tadpole-shaped DNA phages adsorb to bacterial cell walls by use of the tail fibers and tailpins extending from their base plate; (2) Phages equipped with a short noncontractile tail attach when associated with an endoglycosidase; (3) Isometric RNA and filamentous single-stranded DNA phages require the aid of the maturation or A protein (Lindberg, 1973).

It is necessary to consider the anatomy of the phage particle in order to understand how the virus itself actually attaches to a bacterial

receptor site. Anderson (1952), using an electron microscope, showed that bacteriophages are adsorbed by their tail. Therefore, it is the phage tail which carries the organelles responsible for the specific fixation of virus to host cell receptors. With further advances in electron microscopy, it was later revealed that the phage tail is, in fact, a rather complex organelle. The tail fibers appear to be the components responsible for specific attachment of the T-even coliphages. Isolated tail fibers will agglutinate bacteria which are sensitive to the phage (Williams and Fraser, 1956; Kellenberger and Sechaud, 1957; Wildy and Anderson, 1964). Many phages do not normally possess tail fibers and their mechanism of attachment as well as the role played by the base plate and its attached spikes appears to be unknown (Hayes, 1968).

Having considered bacterial receptor sites and bacteriophage anatomy as factors influencing adsorption, one other important factor--the environment--should be mentioned. Inorganic salts must be present which probably neutralize negative charges on bacterium and phage so that initial contact is made (Hayes, 1968). Some bacteriophages require specific divalent cations for adsorption; for instance, phages T4 and lambda require calcium and magnesium respectively. In some cases, adsorption cofactors such as the amino acid tryptophane are required for specific strains of phages T4 and T6 (Hayes, 1968). Tryptophane appears to release the tail fibers from their association with the sheath, enabling them to interact with the bacterial surface. In the absence of tryptophane, the tail fibers of phage T4 can be seen to retract and form a jacket around the sheath and, in the presence of tryptophane, the tail fibers extend (Brenner *et al.*, 1962; Kellenberger *et al.*, 1965). There is also some evidence that zinc complexes in the

bacterial cell may be responsible for tail fiber extension (Kozloff and Lute, 1957).

It appears, then, that the adsorption process is the single most important stage in the growth cycle of a bacteriophage with regard to host specificity. The most important factors affecting host specificity during the adsorption process appear to be the nature and location of bacterial receptor sites, bacteriophage anatomy, and environmental conditions. If the above conditions are not suitable, adsorption cannot take place and the bacteriophage growth cycle cannot continue.

Transduction

In the process of transduction, bacterial DNA is transferred via phage from one bacterial cell to a related, phage-susceptible cell. The transducing phage acts as a vehicle, carrying the DNA inside its protein coat to a susceptible bacterial cell where it may become integrated with the host DNA. As a result, hereditary traits of a previous host bacterial cell may be passed on to form a bacterial cell with a slightly different genetic make-up (Braun, 1965). Transducing phages represent a very small fraction (one in 10^5 to 10^8) of the phage population released either (1) through lysis of sensitive bacterial cells by virulent phages or (2) through the induction of lysogenic, prophage-carrying bacteria (Braun, 1965).

The discovery of transduction in bacterial cells dates back to the early 1950's when Zinder and Lederberg (1952) were trying to determine whether recombination might occur among strains of *Salmonella typhimurium*. They took two different strains, each lacking the ability to synthesize a certain amino acid necessary for growth which the other strain could produce. They took these two strains of bacteria and

placed them within a U-shaped tube, separated by a filter which would allow phage to pass through. An enzyme which would destroy free DNA was added to both sides. If there was a transfer of hereditary material, it would have to be carried by a virus under the protection of its protein coat. A new strain of *Salmonella* developed capable of synthesizing the amino acid it had previously lacked. Genetic material had been exchanged. The process was later given the name transduction.

Two principal types of transduction can be distinguished. General or nonspecific transduction involves phage types which develop from prophages and have the ability to associate themselves with any site on the bacterial chromosome. Restricted or specialized transduction is mediated by phages whose DNA associates itself with only one particular site on the bacterial chromosome. The basic mechanism involved in restricted transduction involves the replacement of a portion of the prophage chromosome with a segment of the bacterial chromosome from a region near the site of prophage attachment; the same mechanism is probably involved in generalized transduction (Adelberg, 1960). However, there appear to be two fundamental differences between transducing phages capable of restricted transduction and those capable of generalized transduction. Phages capable of generalized transduction may attach anywhere on the bacterial chromosome and therefore have an equal probability of picking up any bacterial chromosomal locus and incorporating that locus into the bacterial chromosome adjacent to the allelic site. Phages capable of restricted transduction loosely associate with the bacterial chromosome at a specific site and therefore may transfer a specific chromosomal locus which is not incorporated into the bacterial chromosome but may serve as a template during further chromosomal replication (Jacob and Wollman, 1958). As a result, phages which

carry out generalized transduction appear to produce clones of stable heterogenotes and phages which carry out restricted transduction appear to produce clones of unstable heterogenotes (Adelberg, 1960).

Although transduction is known to occur in more bacterial species than any other method of reassortment of genetic material, little information can be found in the literature concerning its occurrence in nature. Apart from laboratory studies indicating the potential for transduction, what evidence exists which proves that it is a real phenomenon in nature? Jacob (1955) showed that one phage of twenty-three isolated from feces could transduce characters in *Escherichia coli*. However, when considering the low efficiency of transduction (one in 10^6 per character per particle), it is obvious that it could occur only under the rarest conditions, even in the range of phage titers commonly encountered in the intestine (Anderson, 1957).

It is known that a small percentage of strains of members of the Enterobacteriaceae are phage-infected on isolation from feces (Anderson, 1957). Undoubtedly, contact with phage occasionally occurs in the animal intestine. Smith (1948) found three phage types of *Staphylococcus* in different individuals of a herd of cattle and demonstrated that two types grown together would produce the third type. Each parent bacterium was carrying one phage type, but the derived type carried the phages of both parents and was doubly lysogenic.

The most recent evidence of transduction in nature was compiled by Morrison and his co-workers (1978). The results of their investigation demonstrate the ability of phage Fl16 to transduce susceptible strains of *Pseudomonas aeruginosa* under field conditions. Frequencies of transduction of streptomycin resistance ranged from 1.4×10^{-5} to 8.3×10^{-2} transductants per recipient during a 10-day period.

Zinder and Lederberg (1952) demonstrated the phenomenon of transduction and their original observations showed that several different traits could be transduced: nutritional, fermentative, antigenic, and resistance to antibiotics. It is rather interesting to speculate on the ramifications of phage-mediated gene transfer in natural environments. Reanney (1977) relates the "genospecies" concept to transduction and the production of physiologically atypical bacterial strains. In this case, genome size may be kept at a minimum since every individual within a population would not necessarily require a gene for every physiological characteristic of that population.

Gene transfer from extra-environmental microorganisms may play a part in the widespread distribution of antibiotic-resistant microorganisms in natural ecosystems (Aoki, 1975; Parish, 1975). In addition, utilizable substrate transfer, observed in the enterobacteria (Smith and Parsell, 1975), might also occur in other microbial populations in natural environments. With all of the current concern about introduction of artificial bacterial genotypes into the environment, it seems appropriate to investigate the potential for gene transfer via phage in natural ecosystems.

METHODS AND MATERIALS

Description of Sampling Site

A sewage treatment facility in Mattoon (Coles County) Illinois was used in this study. The Mattoon wastewater treatment plant was completed in 1976 in part by a Federal EPA grant-in-aid of approximately $5.3 million. The treatment facility is capable of treating wastewater from a population equivalent to 45,000 people with a hydraulic capacity for treatment of 13 million gallons per day (MGD).

Sewage enters the plant through a 54-inch sewer where it is screened by two mechanical bar screens and pumped to two grit removal tanks. Grit is separated from sewage by controlling the velocity of liquid. After settling, the grit is removed from the bottom of the tanks and transported by truck for disposal at a sanitary landfill. Following grit removal, the sewage is piped into large primary settling tanks. Sludge, grease and scum that float are removed from the surface and pumped to sludge digesters where they are mixed, heated and digested anaerobically. Anaerobic sludge is then placed on sludge drying beds where it is dewatered prior to being hauled by truck to agricultural fields for soil conditioning. Following primary treatment the effluent is pumped to six aerated activated sludge tanks for the biological treatment process. From there it is pumped to two final settling tanks where cell matter and activated sludge are collected at the bottom and returned to the aeration basins or to an aerobic digester. Effluent

from the sludge clarifiers is filtered through a granular media (sand) filter, chlorinated and passed to a chlorine contact tank where it may have a retention time of up to 40 minutes. The disinfected effluent is discharged into Kickapoo Creek. The treatment facility is capable of treating large volumes (up to 26.5 MGD) of wastewater that may enter the plant during periods of high precipitation. This is accomplished by chlorinating excess wastewater and pumping it to four stormwater clarifiers where primary settling occurs before the effluent is discharged into the creek.

Samples were obtained from the following sites at the treatment plant: (1) primary effluent following bar screening and degritting; (2) anaerobic effluent; (3) aerobic (secondary) effluent prior to sand filtration; (4) aerobic (tertiary)effluent after sand filtration; and (5) chlorinated final effluent prior to entry into receiving waters.

Methods

General bacteriophage techniques were those described by Adams (1959). Each sample was collected in a sterile plastic 50 ml screw-capped centrifuge tube. Chlorinated samples were taken in tubes containing 0.1 ml of 10% aqueous sodium thiosulfate to neutralize residual chlorine. As soon as possible after collection, samples were filtered through Millipore HA filters (47 mm diameter 0.45 µm pore diameter) to remove bacterial cells, organic matter and coarse particulates. The sewage filtrate was then mixed with an equal volume of sterile 1% peptone and refrigerated at 4C until use.

Organisms

The host bacterial strains used are described in Appendix A. Stock cultures were maintained on 1.5% nutrient agar slants and stored at 4C. Stocks were transferred monthly. A fresh nutrient agar slant transfer of each culture was prepared and incubated at 35C for 24-36 hours before inoculating broth cultures.

Media

Bacterial broth cultures were raised in flasks of nutrient broth which contained, in 1 liter of distilled water, 8 g of nutrient broth (Difco) and 5 g of sodium chloride. The cultures were grown for 14-16 hours at 30C in a New Brunswick Model G 76 Gyrotory Water Bath Shaker at 140 cpm. For agar overlay plates, base layer agar was prepared by adding 8 g nutrient broth (Difco), 15 g agar (Difco) and 5 g of sodium chloride to 1 liter of distilled water. Soft agar was prepared by adding 12 g nutrient broth (Difco), 12 g agar and 7.5 g sodium chloride to 1 liter of distilled water and dispensing in 2.5 ml amounts in 16 x 150 mm culture tubes. Nine ml dilution blanks were prepared using .1% peptone (Difco). Sterilization of all media was at 121C for 20 minutes in a Castle horizontal autoclave.

Assay of Sewage Samples

Replicate 1 ml samples of undiluted and decimal dilutions of the sewage filtrate were added to sterile 16 mm x 150 mm culture tubes. To each tube, 2-3 drops of a 14-18 hour broth culture of the host bacterium was added. The cell suspension was allowed to stand for 5 minutes at room temperature to allow for phage adsorption. To each tube was added 2.5 ml of soft (seed) agar, equilibrated at 50C in a Napco Model 210 water bath, for a final soft agar concentration of

approximately 0.8% agar at normal nutrient concentration. After mixing sewage filtrate, host cells and soft agar, the agar overlay method was used to determine the plaque-forming unit (PFU) content of the sample. The plates were incubated at 35C for 6-8 hours in a Thelco Model 6 incubator and observed for plaque formation. Plaque number and morphology was determined at this time on the basis of size (small, large), and clarity (clear, halo). Isolated plaques on plates of each sample were examined and the number of plaque morphological classes produced by the sample were determined.

Purification and Preparation of Phage Stocks

Plaques intended for further study were purified and their stocks were prepared by the following method (Dhillon *et al.*, 1970). The center of a selected plaque was stabbed with a sterile, pointed 26 gauge nichrome wire needle and the needle was shaken in 2.0 ml of sterile nutrient broth. Two ten-fold serial dilutions of this suspension were prepared and plated on indicator cells. After two or more successive single-plaque isolations, an isolated plaque was stabbed with the sterile, nichrome needle and inoculated into 2 ml of a 10^{-2} dilution of a 12-hour nutrient broth culture of original host cells. After 8 hours of incubation at 35C, 2 ml of 0.65% agar in 1% tryptone broth (melted and equilibrated at 50C) was added. The tube was labeled by code and stored at 4C.

Spot Tests For Host Range

Phage suspensions for spot tests were prepared by stabbing the stock cultures with a 4 mm diameter inoculating loop and shaking the loop in 2.0 ml of nutrient broth containing 2-3 drops of chloroform (Dhillon *et al.*, 1970). The suspension was agitated vigorously on a

Model K-500-J Vortex Jr. Mixer to suspend the phage and kill bacterial cells. When 4 mm diameter loopfuls of the above suspension were placed on agar overlay plates seeded with indicator bacteria, many phage suspensions were found to be of a titer too low for an accurate host range specificity study. In order to increase phage titer, phage stocks were stabbed with a 4 mm diameter inoculating loop and inoculated into a 10^{-2} dilution of a 12-hour nutrient broth culture of original host cells and incubated at 35C for 8 hours. At this time, 2-3 drops of chloroform were added and the suspension was agitated to kill host bacterial cells. All phage suspensions were then found to produce significant lysis when spot tested against the original host bacterium. Four mm diameter loopfuls of the above suspensions were placed on agar overlay plates seeded with indicator bacteria. Each phage suspension was spot tested against all 36 enteric bacterial strains used in this study.

RESULTS AND DISCUSSION

The sewage treatment process at a wastewater treatment plant has a significant effect on the bacterial virus populations present in sewage effluents. A reduction in bacteriophage numbers would be expected and this is in fact the case (Table 1). Thirty-six different strains of enteric bacteria were used as host organisms in order to determine which bacteria were most susceptible to phages present in sewage effluent. Of the five sites sampled at the sewage treatment facility, the degritted primary effluent at sample site 1 always had the greatest number of phage per ml. This is to be expected since no treatment has taken place other than bar-screening and degritting.

Anaerobic effluent (sample site 2) was only tested against nine host organisms for two reasons. Anaerobic effluent was difficult to collect because it was discharged from the anaerobic digester at irregular intervals. Once collected, the effluent was very difficult to filter due to the large amount of fine, suspended particles present in it. For those nine randomly selected host organisms, the anaerobic effluent showed an average of 98% reduction in phage numbers when compared with sample site 1. This is a great reduction in phage but is not particularly unusual since the effluent at sample site 2 is released from sludge pumped to the anaerobic digester from various stages throughout the treatment process.

TABLE 1. Bacteriophage Populations in Sewage Effluents[a]

Host Organism		Sample Site[b]					% Reduction[d]
		1	2[c]	3	4	5	
Escherichia coli	B	910	14	183	174	82	91
	K12(+)	270	16	9	12	8	97
	K12(-)	172	4	59	32	19	89
	Ill.	0	NR	0	0	0	--
	6601	0	NR	0	0	0	--
	7314	81	NR	7	5	1	99
	Greenberg	0	NR	0	0	0	--
Shigella boydii		92	3	10	12	6	93
S. flexneri		260	2	26	35	22	92
S. sonnei		331	NR	14	21	0	100
Edwardsiella tarda		0	NR	0	0	0	--
Salmonella typhimurium		280	1	101	97	81	71
S. paratyphi	A	1	NR	0	1	0	100
S. arizonae		6	NR	1	2	1	67
S. pullorum	PU	30	NR	1	1	0	100
S. pullorum		0	NR	0	0	0	--
Citrobacter freundii		17	NR	2	2	1	94
Enterobacter cloacae	C	100	NR	16	14	8	92
	C2	88	NR	42	32	22	75
	Greenberg	226	NR	93	66	40	82
	E1	111	NR	38	36	10	91
	E3	17	NR	3	0	0	100
	U1	55	NR	9	5	1	98
E. aerogenes		15	1	3	3	2	87
E. hafniae		3	NR	0	0	1	67
Serratia marcescens		3	NR	0	0	0	100
S. liquefaciens		29	NR	6	7	8	72
Proteus vulgaris		0	NR	0	0	0	--
	S2	0	NR	0	0	0	--
	Simpson	0	NR	0	0	0	--
P. mirabilis		0	NR	0	0	0	--
	Simpson	0	NR	0	0	0	--
P. morganii		2	NR	0	0	0	100
	Simpson	0	NR	0	0	0	--
P. rettgeri		2	0	1	0	1	50
Providencia alcalifaciens		7	0	0	1	0	100

[a] plaques/ml

[b] 1 - primary effluent (after degritting)
2 - anaerobic effluent
3 - aerobic effluent (prior to sand filtration)
4 - aerobic effluent (after sand filtration)
5 - chlorinated final effluent

[c] NR - not run

[d] $100 - \left(\frac{\text{phage titer 5}}{\text{phage titer 1}} \times 100\right)$

Bacteriophages are known to adsorb to sludge particles as well as bacterial cell walls and this could account for some of the tremendous decrease in numbers of plaques observed when titering anaerobic effluent. Another reason for the marked decrease could be the fact that wastewater is heated at 38C during anaerobic digestion, possibly killing large numbers of bacterial and other viruses.

For all host strains, phage titers decreased from sample site 1 when compared to sample site 3. On the average, there was an 80% reduction in phage titer between the two sample sites. This can be attributed to activated sludge stabilization followed by clarification which would sediment cells and adsorbed phage particles.

The smallest decrease in phage titer was observed between sample site 3 (pre-sand filtration) and sample site 4 (post-sand filtration). In some cases the phage titer actually increased somewhat. The phage titer at sample site 4 showed, on the average, an 11% decrease in the number of plaques observed at sample site 3. Sand filtration is undoubtedly responsible for much, if not all, of the decrease observed here, but the level of reduction appears to indicate that sand filtration is relatively ineffective in reducing the number of phages present in sewage effluent.

An average 44% decrease in phage titer was observed between sample sites 4 and 5. This shows directly the effect chlorination alone has on bacteriophage populations in a wastewater treatment plant.

For seven of the thirty-six host organisms tested, 100% mortality of phages was observed. There was never less than a 50% decrease throughout the treatment process and, on the average, a 90% reduction in phage titer was observed from sample site 1 to sample site 5 (Table 1).

E. coli B, *E. coli* K12(+), *E. coli* K12(-), *Salmonella typhimurium* and several species of *Shigella* and *Enterobacter* appear to be the host strains most affected by phages in sewage effluent. *Providencia alcalifaciens* and eight strains of *Proteus* were used as host organisms with almost no plaque formation observed. Due to extensive peritrichous flagellation in many species of *Proteus*, motility was considered as a possible factor masking plaque formation. In order to determine whether swarming was in fact masking plaques before they could be observed, nutrient agar containing 1 percent sucrose and 1 percent Bacto Bile Salts (Coetzee and Hawtrey, 1962) was used to inhibit motility. Petri plates were observed after 4-5 hours of incubation in order to determine whether plaques were forming and immediately being masked by highly motile *Proteus* cells. No plaque formation could be observed at any stage of incubation except for those few plaques observed and recorded (Table 1). Phages specific for *Proteus* species do undoubtedly exist but were only observed on rare occasions throughout the sampling period. Data provided by Coetzee and Hawtrey (1962) indicate that *Proteus* phages may carry genes that, under certain conditions, confer survival advantages on the host organism. The apparent absence of *Proteus* phages in sewage may be a result of phage immunity conferred to the host strains used by the prophage they carry.

In addition to obtaining data on the effects of sewage treatment on bacterial virus populations in wastewater, bacteriophage host range specificity was examined. Twenty-three bacteriophages were isolated in pure culture from host enteric bacteria and no two phages showed the same pattern of specificity when spot-tested against the other host bacteria from which other phages had been isolated (Table 2). This is unusual but not unexpected since plaque morphology was the only criterion used in selecting plaques for pure culture isolation.

TABLE 2. Bacteriophage Host Range Specificity

Host Organism	Bacteriophage Isolate																					
	ECBS	ECBL	K12NS	K12NL	K12PS	K12PL	EC73SC	EC73LC	SBSH	SFSC	SSLH	SSSC	STSC	SPSC	SPASC	CFSC	EC2SC	ECE1SC	ECE3SC	EASC	EHSC	SLSH	SLLH
Escherichia coli B	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-
K 12(-)	-	-	+	+	-	-	+	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-
K 12(+)	-	+	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	+	-	-	-
7314	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Shigella boydii	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. flexneri	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
S. sonnei	-	+	+	+	+	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-	-	+	-
Salmonella typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
S. pullorum PU	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. paratyphi A	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Citrobacter freundii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-

\+ = lysis
\- = no lysis

TABLE 2. Bacteriophage Host Range Specificity (contd.)

Host Organism	Bacteriophage Isolate																					
	ECBS	ECBL	K12NS	K12NL	K12PS	K12PL	EC73SC	EC73LC	SBSH	SFSC	SSLH	SSSC	STSC	SPSC	SPASC	CFSC	EC2SC	ECE1SC	ECE3SC	EASC	EHSC	SLSH	SLLH
Enterobacter cloacae C	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-
C2	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-
E1	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-
E3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
UI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
E. aerogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
E. hafnia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Serratia marcescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. liquefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Proteus vulgaris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Providencia alcalifaciens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

\+ = lysis
\- = no lysis

As a control, each phage isolate was spot-tested against its original host and in every case significant lysis was visible. Five enterics, from which no phages were isolated, were also spot-tested against all phage isolates. These five include *E. cloacae* C, *E. cloacae* UI, *S. marcescens*, *P. vulgaris* and *P. alcalifaciens*. Both strains of *E. cloacae* showed lysis but only when tested against other *E. cloacae* phages. *S. marcescens*, *P. vulgaris* and *P. alcalifaciens* showed no lysis when tested against all twenty-three phage isolates (Table 2).

Phage ECE3SC (isolated from *E. cloacae E3*) possessed the ability to lyse seven of the twenty-two host bacteria it was spot-tested against, while other phages showed lysis only when tested against their original host. These included SBSH, SPSC, EASC, EHSC, SLSH and SLLH. (The original host and plaque morphology for each of these isolates is listed in Appendix B). *E. coli* K12(+) was attacked by 13 phage isolates, more than any other host organism, and five host enterics were lysed only by the phages originally isolated from them. These included *S. boydii*, *S. pullorum* PU, *E. aerogenes*, *E. hafniae* and *S. liquefaciens*.

Having considered the effects of sewage treatment on bacteriophage populations and the host range specificity exhibited by those phages isolated, the potential for transduction via phage can now be considered (Table 3). Strain specificity was exhibited by only 13% (3 of 23) of all phages isolated. Species specificity was exhibited by 30% (7 of 23) of the phage isolates. (This percentage includes the 13% which were strain specific.) Thirty-five percent (8 of 23) of all phage isolates are specific for a genus of bacterium. (This percentage includes all strain and species specific phages.) Sixty-one percent (14 of 23) of

TABLE 3. Potential For Transduction Among Enteric Strains Studied

I. STRAIN SPECIFICITY

Phage	Original Host
SFSC	*Shigella flexneri*
EHSC	*Enterobacter hafnia*
SLLH	*Serratia liquefaciens*

II. SPECIES SPECIFICITY

Phage	Original Host
K12PL	*Escherichia coli* K12 (+)
EC73LC	*E. coli* 7314
EC2SC	*Enterobacter cloacae* C2
ECE1SC	*E. cloacae* E1

III. GENUS SPECIFICITY

Phage	Original Host
SPSC	*Salmonella pullorum*

IV. TRIBE SPECIFICITY

Phage	Original Host
K12NS	*Escherichia coli* K12 (-)
K12PS	*E. coli* K12 (+)
EC73SC	*E. coli* 7314
SBSH	*Shigella boydii*
SSLH	*S. sonnei*
SSSC	*S. sonnei*

TABLE 3. Potential For Transduction Among Enteric Strains Studied (cont.)

V. INTERTRIBE SPECIFICITY

A. *ESCHERICHIEAE - KLEBSIELLEAE*

Phage	Original Host
ECBS	*Escherichia coli B*
ECBL	*E. coli B*
ECE3SC	*Enterobacter cloacae E3*
EASC	*E. aerogenes*
SLSH	*Serratia liquefaciens*

B. *ESCHERICHIEAE - SALMONELLEAE*

Phage	Original Host
K12NL	*Escherichia coli K12 (-)*
SPASC	*Salmonella paratyphi A*
CFSC	*Citrobacter freundii*

C. *KLEBSIELLEAE - SALMONELLEAE*

Phage	Original Host
STSC	*Salmonella typhimurium*

all phages isolated were specific for a particular tribe of the bacterial family Enterobacteriaceae. (This percentage includes phages which showed specificity at the genus, species and strain levels.) Thirty-nine percent (9 of 23) of all bacteriophages isolated showed a specificity capable of crossing tribal boundaries. Of these, 56% showed the potential to transfer genes between the Escherichieae and Klebsielleae. Thirty-three percent showed the potential to transfer genes between the Escherichieae and Salmonelleae and 11% showed the potential to transfer genes between the Klebsielleae and Salmonelleae.

In order for transduction to be possible in a natural environment, several factors must be considered. The absence of strain specificity is required in order for transduction to occur. In addition, there must be an adequate number of phage since the efficiency of transduction is low (one in 10^6 per character per particle). This research did not determine what the actual population density of any one phage isolate was in the sewage effluents sampled but, assuming that an adequate number of host cells were present, the probability of transduction occurring is low even when considering the range of phage titers commonly encountered in the intestine (Anderson, 1957). Since only 3 of 23 phage isolates were strain specific, the potential for transduction to occur does in fact exist.

Reanney (1977) believes that DNA transfer is a prime vehicle of adaptive change in all prokaryotes from the standpoint of comparative bacterial genetics and ecology. Zinder and Lederberg (1952) showed that several different traits could be transduced: nutritional, fermentative, antigenic and antibiotic resistance. With the potential for transduction between tribes, it is foreseeable that pathogenicity might also be a transferrable trait to consider, especially between the

relatively non-pathogenic tribes and the enteric pathogens. In summary, it would appear that intercellular phage-mediated DNA transfer might constitute a valuable device when considering the adaptive flexibility of microorganisms in natural environments.

CONCLUSIONS

1. Bacteriophage populations are adversely affected by sewage treatment. At least 50 percent of a bacterial virus population may be lost due to wastewater treatment and, in some cases, 100 percent mortality may be observed.

2. An average of 44 percent reduction can be attributed to the effects of chlorination.

3. Activated sludge stabilization may be responsible for up to 80 percent reduction in phage titer.

4. *Proteus* species appear to produce very few plaques when tested using the agar overlay method. This does not necessarily mean that *Proteus* phages are not present in sewage effluents.

5. *E. coli B*, *E. coli* K12(+), *E. coli* K12(-), *S. typhimurium* and several species of *Shigella* and *Enterobacter* appear to be the host enterics most affected by phages in sewage effluent.

6. An *E. cloacae* phage isolate possessed the ability to lyse more host enterics than any other phage isolate.

7. Thirty-nine percent of all phage isolates exhibited intertribal specificity while only 13 percent of the phages isolated were specific for a particular strain of bacteria.

APPENDIX A

BACTERIAL CULTURES

APPENDIX A

BACTERIAL CULTURES

		Dextrose	Gas	Lysine	Ornithine	H_2S	Indole	Lactose	Culcitol	Phenylalanine	Urea	Citrate
Escherichia coli B		+[a]	+	+	S[b]	-[c]	+	+	-	-	-	-
E. coli	K12(+)	+	+	-	+	-	+	+	+	-	-	-
E. coli	K12(-)	+	+	-	-	-	+	-	+	-	-	-
E. coli	Ill.	+	+	+	-	-	+	+	-	-	-	-
E. coli	6601	+	+	+	-	-	+	+	+	-	-	-
E. coli	7314	+	+	-	-	-	+	+	-	-	-	-
E. coli	Greenberg	+	+	+	+	-	+	+	+	-	-	-
Shigella boydii		+	-	-	-	+	-	-	-	-	-	-
S. flexneri		+	-	+	+	-	-	-	-	-	-	-
S. sonnei		+	-	+	-	-	-	+	-	-	-	-
Edwardsiella tarda		+	+	+	+	+	+	-	-	-	-	-
Salmonella typhimurium		+	+	+	+	+	-	-	+	-	-	+
S. paratyphi A		+	+	-	-	+	-	+	+	-	-	+
S. arizonae		+	+	+	+	+	-	-	-	-	-	-
S. pullorum	PU	+	+	+	+	+	-	-	-	-	-	-
S. pullorum		+	+	+	+	S	-	-	-	-	-	-
Citrobacter freundii		+	+	-	+	+	-	+	+	-	S	+
Enterobacter cloacae C		+	+	-	+	-	-	+	-	-	-	+

APPENDIX A

BACTERIAL CULTURES (cont.)

		Dextrose	Gas	Lysine	Ornithine	H_2S	Indole	Lactose	Dulcitol	Phenylalanine	Urea	Citrate
E. cloacae	C2	+	+	-	+	-	-	+	-	-	-	+
E. cloacae	Greenberg	+	+	-	+	-	-	+	-	-	-	+
E. cloacae	E1	+	+	-	+	-	-	+	-	-	-	+
E. cloacae	E3	+	+	-	S	-	-	+	-	-	-	+
E. cloacae	UI	+	+	-	+	-	-	+	-	-	-	+
E. aerogenes		+	+	+	+	-	-	+	-	-	-	+
E. hafniae		+	+	+	+	-	-	-	-	-	-	-
Serratia marcescens		+	+	+	+	-	-	+	-	-	-	+
S. liquefaciens		+	+	+	+	-	-	+	-	-	-	+
Proteus vulgaris		+	+	-	-	+	+	-	-	+	+	-
P. vulgaris	S2	+	-	-	-	+	+	+	-	+	+	+
P. vulgaris	Simpson	+	+	+	+	+	+	-	-	+	+	-
P. mirabilis		+	+	-	+	+	-	-	-	+	+	+
P. mirabilis	Simpson	+	+	-	+	+	-	+	-	+	+	S
P. morganii		+	+	-	+	-	+	-	-	+	+	-
P. morganii	Simpson	+	+	-	+	-	-	+	+	+	+	-
P. rettgeri		+	-	-	-	-	+	+	+	+	+	+
P. alcalifaciens		+	-	-	-	-	+	-	-	+	-	+

[a] + positive after 24 hrs.
[b] S positive after 48 hrs.
[c] - negative after 48 hrs.

APPENDIX B

BACTERIOPHAGE ISOLATES

APPENDIX B

BACTERIOPHAGE ISOLATES

Host Organism		Phage Isolate	Plaque Morphology
Escherichia coli B		ECBS	small, clear
		ECBL	large, clear
E. coli	K12(+)	K12PS	small, clear
		K12PL	large, clear
E. coli	K12(-)	K12NS	small, clear
		K12NL	large, clear
E. coli	7314	EC73SC	small, clear
		EC73LC	large, clear
Shigella boydii		SBSH	small, halo
S. flexneri		SFSC	small, clear
S. sonnei		SSSC	small, clear
		SSLH	large, halo
Salmonella typhimurium		STSC	small, clear
S. pullorum	PU	SPSC	small, clear
S. paratyphi	A	SPASC	small, clear
Citrobacter freundii		CFSC	small, clear
Enterobacter cloacae	C2	EC2SC	small, clear
E. cloacae	E1	ECE1SC	small, clear
E. cloacae	E3	ECE3SC	small, clear
E. aerogenes		EASC	small, clear
E. hafniae		EHSC	small, clear
Serratia liquefaciens		SLSH	small, halo
		SLLH	large, halo

LITERATURE CITED

Adams, M. 1959. Bacteriophages. Interscience. New York.

Adelberg, E. 1960. Papers on Bacterial Genetics. Little, Brown and Company, Limited. Boston and Toronto.

Anderson, E. 1957. The relations of bacteriophages to bacterial ecology. In Microbial Ecology. Seventh Symposium of The Society For General Microbiology. Cambridge University Press. New York.

Anderson, T. F. 1952. Stereoscopic studies of cells and viruses in the electron microscope. Am. Naturalist. 86 : 91-100.

Anderson, T. F. 1953. The morphology and osmotic properties of bacteriophage systems. Cold Spring Harbor Symposia Quant. Biol. 18 : 197-203.

Aoki, T. 1975. Effects of chemotherapeutics on bacterial ecology in the water of ponds and the intestinal tracts of cultured fish. Ayu (*Plecoglossus altivelis*). Jpn. J. Microbiol. 19 : 7-12.

Barrington, L. and L. Kozloff. 1954. Action of T_2r^+ bacteriophage on host-cell membranes. Science. 120 : 110-111.

Braun, W. 1965. Bacterial Genetics. W. B. Saunders Company. Philadelphia and London.

Brenner, S., S. Champe, G. Streisinger and I. Barnett. 1962. On the interaction of adsorption cofactors with bacteriophages T2 and T4. Virology. 17 : 30-39.

Burnet, F. M. and D. Lush. 1936. Induced lysogenicity and mutation of bacteriophage within lysogenic bacteria. Australian Jour. Exp. Biol. and Med. Sci. 14 : 27-38.

Coetzee, J. N. and A. O. Hawtrey. 1962. A change in phenotype associated with the bacteriophage carrier state in a strain of *Proteus mirabilis*. Nature. 194 : 1196-1197

Cohen, S. S. 1949. Growth requirements of bacterial viruses. Bacteriol. Rev. 13 : 1-24.

Dhillon, T. S., Y. S. Chan, S. M. Sun and W. S. Chau. 1970. Distribution of coliphages in Hong Kong sewage. Appl. Microbiol. 20 : 187-191.

Doermann, A. H. 1953. The vegetative state in the life cycle of bacteriophage: Evidence for its occurrence and its genetic characterization. Cold Spring Harbor Symp. Quant. Biol. 18 : 3-11.

Foster, R. A. C. 1948. An analysis of the action of proflavine on bacteriophage growth. J. Bacteriol. 56 : 795-809.

Hayes, W. 1968. The Genetics of Bacteria and Their Viruses, 2nd ed. John Wiley and Sons, Inc., New York.

d'Herelle, F. 1917. Sur un microbe invisible antagonistic des bacilles dysenterique. C. R. Acad. Sci. Paris. 165 : 373-375.

d'Herelle, F. 1926. The Bacteriophage And Its Behavior. Williams and Wilkins. Baltimore.

Hershey, A. D. and M. Chase. 1952. Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol. 36 : 39-56.

Hewitt, L. 1953. Influence of bacteriophage on bacterial variation and evolution. In Adaptation In Micro-Organisms. Third Symposium Of The Society For General Microbiology. Cambridge University Press. New York.

Jacob, F. 1955. Transduction of lysogeny in *Escherichia coli*. Virology. 1 : 207-220.

Jacob, F., A. Lwoff, L. Siminovitch and E. Wollman. 1953. Definition de quelques termes relatifs a' la lysogenie. Ann. Inst. Pasteur. 84 : 222-224.

Jacob, F. and E. L. Wollman. 1958. The relationship between the prophage and the bacterial chromosome in lysogenic bacteria. In Recent Progress in Microbiology, G. Tunevall, Ed., p. 15. Almquist and Wiksell. Stockholm.

Jawetz, E., J. Melnick and E. Adelberg. 1974. Review of Medical Microbiology, 11th ed. Lange Medical Publ., Los Altos, Ca.

Kalmanson, G. and J. Bronfenbrenner. 1942. Evidence of serological heterogeneity of polyvalent "pure line" bacteriophage. J. Immunol. 45 : 13-19.

Kellenberger, E., A. Bolle, E. Boy De La Tour, R. Epstein, N. Franklin, N. Jerne, A. Reale-Scafati, J. Sechaud, I. Bendet, D. Goldstein and N. Lauffer. 1965. Functions and properties related to the tail fibers of bacteriophage T4. Virology. 26 : 419-440.

Kellenberger, E. and J. Sechaud. 1957. Electron microscopical studies of phage multiplication. II. Production of phage-related structures during multiplication of phages T2 and T4. Virology. 3 : 256-274.

Kozloff, L. and M. Lute. 1957. Viral invasion. II. The role of zinc in bacteriophage invasion. J. Biol. Chem. 228 : 529-536.

Lazarus, A. S. and J. B. Gunnison. 1947. The action of *Pasteurella pestis* bacteriophage on strains of *Pasteurella*, *Salmonella* and *Shigella*. J. Bacteriol. 53 : 705-714.

Lindberg, A. 1973. Bacteriophage receptors. Ann. Rev. Microbiol. 27 : 205-241.

Lisbonne, M. and L. Carrère. 1922. Antagonisme microbien et lyse transmissible du bacille de shiga. C. R. Soc. Biol. Paris. 86 : 569-570.

Morrison, W. D., R. Miller and G. Sayler. 1978. Frequency of F116-mediated transduction of *Pseudomonas aeruginosa* in a freshwater environment. Appl. and Environ. Microbiol. 36 : 724-730.

Parish, J. H. 1975. Transfer of drug resistance to myxococcus from bacteria carrying drug resistance factors. J. Gen. Microbiol. 87 : 198-210.

Reanney, D. 1977. Gene transfer as a mechanism of microbial evolution. Bio Science 27 : 340-344.

Salton, M. R. J. 1960. Surface layers of the bacterial cell. In I. Gunsalus and R. Stanier (ed.) The Bacteria, Vol I : Structure. Academic Press, New York.

Smith, H. W. 1948. Investigations on the typing of staphylococci by means of bacteriophage. J. Hyg. Camb. 46 : 82-89.

Smith, H. W. and Z. Parsell. 1975. Transmissable substrate-utilizing ability in enterobacteria. J. Gen. Microbiol. 87 : 129-140.

Stent, G. 1963. Molecular Biology of Bacterial Viruses. W. H. Freeman, San Francisco.

Twort, F. W. 1915. An investigation on the nature of ultra-microscopic viruses. Lancet 2 : 1241-1243.

Weibull, C. 1953. The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme. J. Bacteriol. 66 : 688-702.

Weidel, W. 1955. L-Gala-D-Mannoheptose, a building block of bacterial cell walls. Hoppe-Seyler's Z. Physiol. Chem. 299 : 253-257.

Weidel, W., G. Koch and K. Bobosch. 1954. The receptor substance for phage T5. I. Extraction and purification from *Escherichia coli*. Physical, chemical, and functional properties. Z. Naturforsch. 9b : 573-579.

Wildy P. and T. F. Anderson. 1964. Clumping of susceptible bacteria by bacteriophage tail fibres. J. Gen Microbiol. 34 : 273-283.

Williams, R. C. and D. Fraser. 1953. Morphology of the seven T-bacteriophages. J. Bacteriol. 66 : 458-464.

Williams, R. C. and D. Frazer. 1956. Structural and functional differentiation in T2 bacteriophage. Virology. 2 : 289-307.

Zinder, N. D. and W. F. Arndt. 1956. Production of protoplasts of *Escherichia coli* by lysozyme treatment. Proc. Natl. Acad. Sci. 42 : 586-590.

Zinder, N. D. and J. Lederberg. 1952. Genetic exchange in *Salmonella*. J. Bacteriol. 64 : 679-699.